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Agent's address: Broadgate House
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Agent's ADP number: 745002

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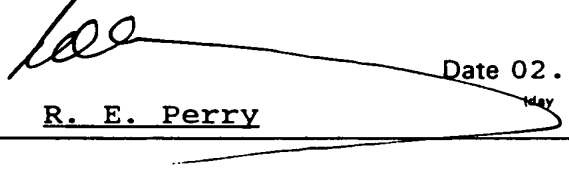
Signed  Date 02. 07. 93 (day month year)

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PROTEIN KINASES

This invention is related to the one or more inventions described in British Patent Applications Nos. 9224057.1, filed 17th November 1992, 9304677.9 and 9304680.3, both filed 8th March 1993, and 9311047.6, filed 28th May 1993. In particular, this invention relates to nucleotides, proteins obtained by expression therefrom and antibodies raised to peptides derived from the sequence, e.g. by the means described in the Application No. 9304680.3.

Summary: Mouse cDNA clones encoding putative serine/threonine kinase receptors denoted activin receptor-like kinase (ALK)-3 and -6 were obtained from a 12 day mouse embryo cDNA library. The cDNA clones for ALK-3 and -6 encode complete proteins of 532 and 502 amino acids, respectively. They have hydrophilic cysteine-rich ligand-binding domains, followed by single hydrophobic transmembrane regions and C-terminal intracellular portions containing putative serine/threonine kinase domains. The amino acid sequences of ALK-3 and -6 are very similar with 59% and 85% sequence identities in the extracellular cysteine-rich domains and intracellular serine/threonine kinase domains, respectively. Expression of mRNA for mouse ALK-3 was observed in the spleen, whereas ALK-6 mRNA was found in the brain. These results suggest that ALK-3 and -6 bind closely related ligands, but that they have different functional roles *in vivo*.

Transforming growth factor-β (TGF-β) is a family of multifunctional proteins, including TGF-β1, -β2, and -β3 in mammals. They regulate the growth and differentiation of many different cells, and stimulate the production of extracellular matrix proteins (1). TGF-βs belong to a larger superfamily of structurally related proteins, which includes activins and inhibins, Müllerian inhibiting substance, and bone morphogenetic proteins (BMPs) or osteogenic proteins (2, 3). Proteins in the TGF-β superfamily regulate the cellular proliferation and differentiation, and play important roles at the different stages during the development.

TGF-βs exert their effects through binding to specific cell surface receptors (4, 5). Although TGF-β receptors of several different size classes have been reported, the type I (53 kDa) and type II (70 kDa) receptors have been shown to be most important for signal transduction (6, 7). TGF-β receptors type I and type II are indispensable for signal transduction, and they have been shown to form a heteromeric complex on the cell surface (8). In analogy with the TGF-β receptor system, classes of receptors of similar sizes, i.e. receptors type I (50-60 kDa) and type II (70-80 kDa), have been reported also for activin A and BMP-4 (9, 10).

Molecular cloning of the type II receptors for activin (ActR-II and ActR-IIB) (11-13) and for TGF-β (TβR-II) (14) revealed that they have sequence homology with each other and with a previously identified *C. elegans* gene product, Daf-1 (15). These

molecules have putative serine/threonine kinase domains in their intracellular portions, which suggests that phosphorylation on serine/threonine residues is involved in signal transduction. Using a polymerase chain reaction (PCR)-based strategy, we have recently obtained human cDNA clones encoding novel putative serine/threonine kinase receptors, denoted activin receptor-like kinases (ALK)-1 to -5 (16 and Franzén et al., submitted for publication). The ALKs are more similar with each other than with ActR-II and -IIB, TβR-II, and Daf-1, and thus, they form a subfamily among the putative serine/threonine kinase receptors. Ligands for ALKs remain to be determined. A recent report showed that Tsk 7L, a mouse couterpart of human ALK-2, binds TGF-β1 and forms a cross-linked complex with a size similar to that of the TGF-β type I receptor (17). In this communication, we report the cloning of another serine/threonine kinase receptor, termed ALK-6, from a mouse embryo cDNA library. Interestingly, mouse ALK-6 is structurally very similar to human and mouse ALK-3, but their expression patterns are very different.

MATERIALS AND METHODS

Cloning of cDNAs for Mouse ALK-3 and ALK-6. As a probe for screening a cDNA library, a PCR recombinant 11.1 (16), which corresponds to the kinase domain of human ALK-4, was used. A 12 day mouse embryo λEX*lox* cDNA library (Novagen, Madison, WI) was screened with the 11.1 probe labeled by the Megaprime labeling system (Amersham, U.K.). Hybridization to nitrocellulose replica filters was performed as described previously (18). The filters were washed two times with 2 x SSC (1 x SSC is 15 mM sodium citrate, 150 mM NaCl) and 0.1% SDS at 37°C for 15 min, and two times with 0.5 x SSC and 0.1% SDS at 55°C for 15 min. Purification of bacteriophages were performed as previously described (18). Twenty-four positive clones were obtained. They were analyzed by Southern blot hybridization using specific probes for ALK-1 to -4. In order to obtain the *5'* part of mouse ALK-3, the cDNA library was screened with a probe corresponding to nucleotides 79-824 of human ALK-3 (16). Nucleotide sequencing was performed on both strands (19) using Sequenase (U.S. Biochemical Corporation, Cleveland, OH) and specific oligonucleotide primers. Compressions were resolved using 7-deaza-GTP (U.S. Biochemicals). DNA sequences were analyzed by DNA STAR computer program (DNA STAR, Ltd. U.K.).

Southern Blot Hybridization. DNAs from positive clones were digested with *Eco*RI and *Hin*dIII, electrophoretically separated on a 1.3% agarose gel and transferred to nitrocellulose filters as described (20). The filters were then hybridized with specific probes (16) for human ALK-1 (nucleotides 288-670), ALK-2 (nucleotides 1-581), ALK-3 (nucleotides 79-824) or ALK-4 (nucleotides 1178-1967).

Northern Blot Hybridization. A multiple mouse tissue blot was obtained from Clontech (Palo Alto, CA). The filter was hybridized with probes for mouse ALK-3 and ALK-6. The *Eco*RI - *Pst*I restriction fragment, corresponding to nucleotides 79-1100 of ALK-3, and the *Sac*I - *Hpa*I fragment, corresponding to nucleotide 57-720 of ALK-6, were used as probes. Hybridization was performed with ^{32}P-labeled probes at 42°C overnight in 50% formamide, 5 x SSC, 0.1% SDS, 50 mM sodium phosphate, pH 7.0, 5 x Denhardt's solution, and 0.1 mg/ml salmon sperm DNA. The filter was washed at 65°C, two times for 30 min in 2.5 x SSC, 0.1% SDS, and two times for 30 min with 0.3 x SSC, 0.1% SDS. The filter was then subjected to autoradiography. Stripping of the blot was performed by incubation in distilled water water at 90-100°C for 20 min.

RESULTS AND DISCUSSION

Cloning of Mouse ALK-6 cDNA. By screening the 12 day mouse embryo cDNA library using a probe from the kinase domain of ALK-4 under low stringency hybridization conditions, we obtained 20 positive clones. DNAs from these clones were analyzed by Southern blot hybridization in order to investigate whether they were the mouse homologs of human ALK-1 to -4. Seven clones hybridized very strongly with the ALK-2 probe, seven clones hybridized with the ALK-3 probe, and two clones hybridized with the ALK-4 probe. None of them hybridized to ALK-1 specific probes. Four clones hybridized to the probe corresponding to the conserved kinase domain of ALK-4, but not to probes from more divergent parts of ALK-1 to -4. Analysis of these clones revealed that they have an identical sequence, which was different from those of ALK-1 to -5; therefore, the novel clone was termed ALK-6. The longest clone ME 6 with a 2.0 kb insert was completely sequenced.

Sequence of Mouse ALK-6. Sequencing of ME 6 yielded a 1952 bp fragment consisting of an open reading frame of 1509 bp (503 amino acids), flanked by a 5' untranslated sequence of 186 bp, and a 3' untranslated sequence of 157 bp. The nucleotide and predicted amino acid sequences are shown in Fig. 1. No polyadenylation signal was found in the 3' untranslated region of ME 6, indicating that the cDNA was internally primed in the 3' end. Only one ATG codon was found in the 5' part of the open reading frame, which fulfilled the rules for translation initiation (21), and was preceded by an in-frame stop codon at nucleotides 163-165. However, a typical hydrophobic leader sequence was not observed at the N-terminus of the translated region. Since there is no ATG codon and putative hydrophobic leader sequence, this ATG codon is likely to be used as a translation initiation site. It remains to be determined how efficient the mouse ALK-6 is transported to the cell membrane. Signal peptidase possibly cuts between amino acids 13 and 14, which is a preferrable site according to the von Heijne algorithm (22).

Similar to other ALKs, ALK-6 has a relativly short extracellular domain, followed by a transmembrane domain, and an intracellular putative serine/threonine kinase domain. The extracellular domain has a cysteine-rich domain, which is likely to bind ligands. The serine/threonine kinase receptors, thus far reported, have one or more potential N-glycosylation sites (11-16). However, the mouse ALK-6 does not have any potential N-glycosylation sites.

The intracellular domain of ALK-6 consists almost entirely of a putative kinase domain. A consensus sequence for the binding of ATP (Gly-X-Gly-X-X-Gly in subdomain I, followed by a lysine residue further downstream in subdomain II;

nomenclature according to Ref. 23) is found. Analysis of the amino acid sequences in subdomains VI and VIII, which are most useful to predict the specificity of amino acid phosphorylation (23), indicates that ALK-6 is a serine/threonine kinase. Two kinase inserts were observed between subdomains VIA and VIB, and between subdomains X and XI. The amino acid sequence of mouse ALK-6 is most similar to human ALK-3 among the serine/threonine kinase receptors (16). To rule out the possibility that ALK-6 is the mouse counterpart of ALK-3, we cloned a mouse cDNA for ALK-3 and compared it with the mouse ALK-6.

Cloning of Mouse ALK-3 cDNA. Southern blot analysis revealed that a clone termed ME-7 hybridized with the human ALK-3 probe, however, nucleotide sequencing revealed that this clone was incomplete, and lacked the 5' part of the translated region. Therefore, the same cDNA library was screened by a probe corresponding to the extracellular domain of human ALK-3, and one positive clone, ME-D, was obtained. The clone was isolated and the sequence was analyzed. Although ME-D was incomplete in the 3' end of the translated region, ME-7 and ME-D overlapped and together covered the complete sequence of mouse ALK-3. The predicted amino acid sequence of mouse ALK-3 is very similar to the human sequence; only 8 amino acid residues differ (98% identity) (Fig. 2).

Comparison of Mouse ALK-3 and ALK-6. The amino acid sequences of mouse ALK-3 and -6 are 71% identical; the identities in the kinase domains and the cysteine-rich putative ligand-binding domains are 85% and 59%, respectively. The amino acid sequence similarities in the extracellular cysteine-rich domains of other ALKs and ActR-II, TβR-II and Daf-1 are lower (less than 40%). High sequence similarity in this domain has been reported for ActR-II and ActR-IIB (64% identity), which bind the same ligand. Thus, ALK-3 and ALK-6 are closely related molecules in the serine/threonine kinase receptor family, and may bind the same or closely related ligand(s). The calculated molecular weights of the primary translated products of mouse ALK-3 and -6, without the putative signal sequences, are 57,447 and 55,576, respectively. Therefore, the molecular weights are closer to those of type I receptors than type II receptors. The intracellular portions of ALK-3 and -6 are highly conserved compared to the extracellular domains. However, the intracellular juxtamembrane domain and at the kinase insert between subdomains X and XI show relatively high divergence. Whether these portions functions, e.g. in the association with downstream components in the signal transduction pathway, remains to be elucidated.

Expression of mRNA for Mouse ALK-3 and ALK-6. The distribution of mRNA for mouse ALK-3 and -6 in various mouse tissues was determined by Northern

blot analysis (Fig. 3). In order to avoid cross-hybridization, probes corresponding to the 5' untranslated and extracellular regions were used. Using the probe for mouse ALK-3, a 1.1 kb transcript was found only in spleen. By reprobing the blot with the ALK-6 specific probe, a transcript with 7.2 kb was found in brain. A weak band was also seen in lung; however, bands were not seen in the other tissues tested. Most of the serine/threonine kinase receptors are widely expressed, exemplified by ALK-2, and -4, and TβR-II (14, 16). In contrast, the expression profile of human ALK-3 is relatively restricted, and fragments of 4.4 and 7.9 kb were found most abundantly in skeletal muscle (16). Expression in spleen was not tested for the human ALK-3. The transcript size for mouse ALK-3 is smaller than that of the cloned cDNA. Larger size transcripts may possibly be seen in other tissues which were not tested in the present study, and/or at other developmental stages. As the ALK-3 coding region is 1596 bp, the 1.1 kb transcript is possibly derived from a differently spliced mRNA. The functional significance remains to be elucidated. Difference in mRNA splicing in the region coding for the extracellular domain may lead to the production of soluble binding protein. Difference in the intracellular domain may lead to a truncated cell surface receptor that possibly acts in a dominant negative fashion. Different expression profiles between ALK-3 and -6 suggest different roles of these receptors *in vivo*. Growth regulatory factors have been shown to act in autocrine and/or paracrine fashion. If this is also the case for ALK-3 and -6, they may bind proteins with restricted expression profiles. For example, certain members in the BMP family were shown to be produced with highly limited expression patterns (24-26). Further investigation are in progress in order to identify the ligand(s) for ALK-3 and -6.

Acknowledgments: We thank Kari Alitalo at Helsinki University, Finland for the cDNA library. We also thank Christer Wernstedt for preparing oligonucleotides. Peter ten Dijke is supported by an EMBO fellowship. Nucleotide sequences of mouse ALK-3 and -6 are deposited in GenBank.

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Figure Legends

Fig. 1. Nucleotide and deduced amino acid sequences of mouse ALK-6. The putative signal sequence and transmembrane domain are overlined over the amino acid sequence. Cysteine residues found in the extracellular domain are boxed. The borders of the kinase domain are marked by arrows. The stop codon which ends the open reading frame is shown by an asterisk. The in-frame stop codon is underlined.

Fig. 2. Comparison of the amino acid sequences of human and mouse ALK-3 and mouse ALK-6. Identical amino acids are boxed and conserved cysteine residues in the extracellular domains are shaded. Potential N-glycosylation sites for human and mouse ALK-3 are shown by a thick line. Putative cleavage sites by signal peptidase are indicated by A. Putative transmembrane domains for human and mouse ALK-3 are double overlined. The borders of the kinase domains are indicated by arrows. Kinase subdomains (23) are indicated by roman numerals. The two kinase inserts are underlined (thin lines).

Fig. 3. Northern blot analysis of mouse ALK-3 and -6 mRNA expression in different mouse tissues. A multiple mouse tissue blot (Clontech) was hybridized with probes for mouse ALK-3 (A) and ALK-6 (B). Size markers are indicated on the right. Specific bands are indicated by arrows on the left.

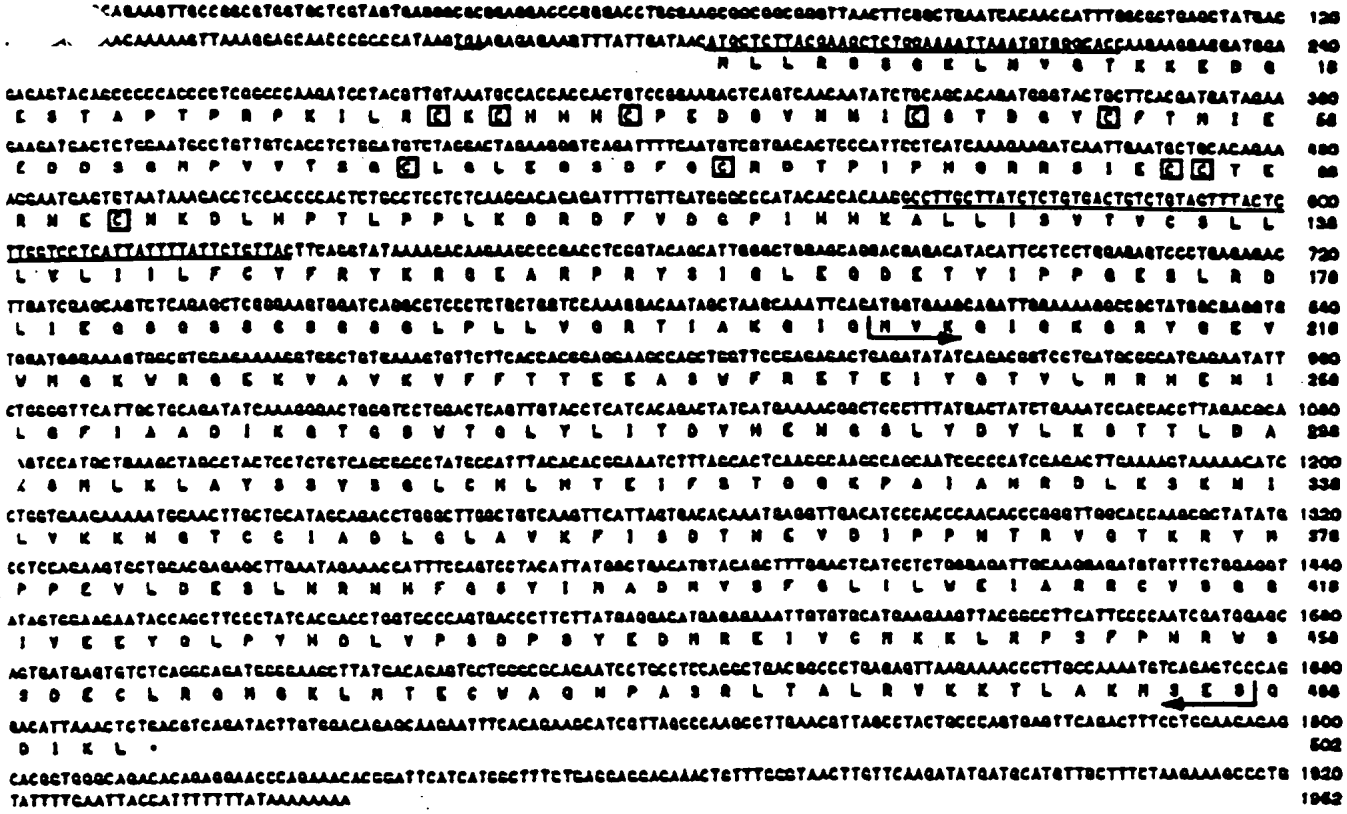
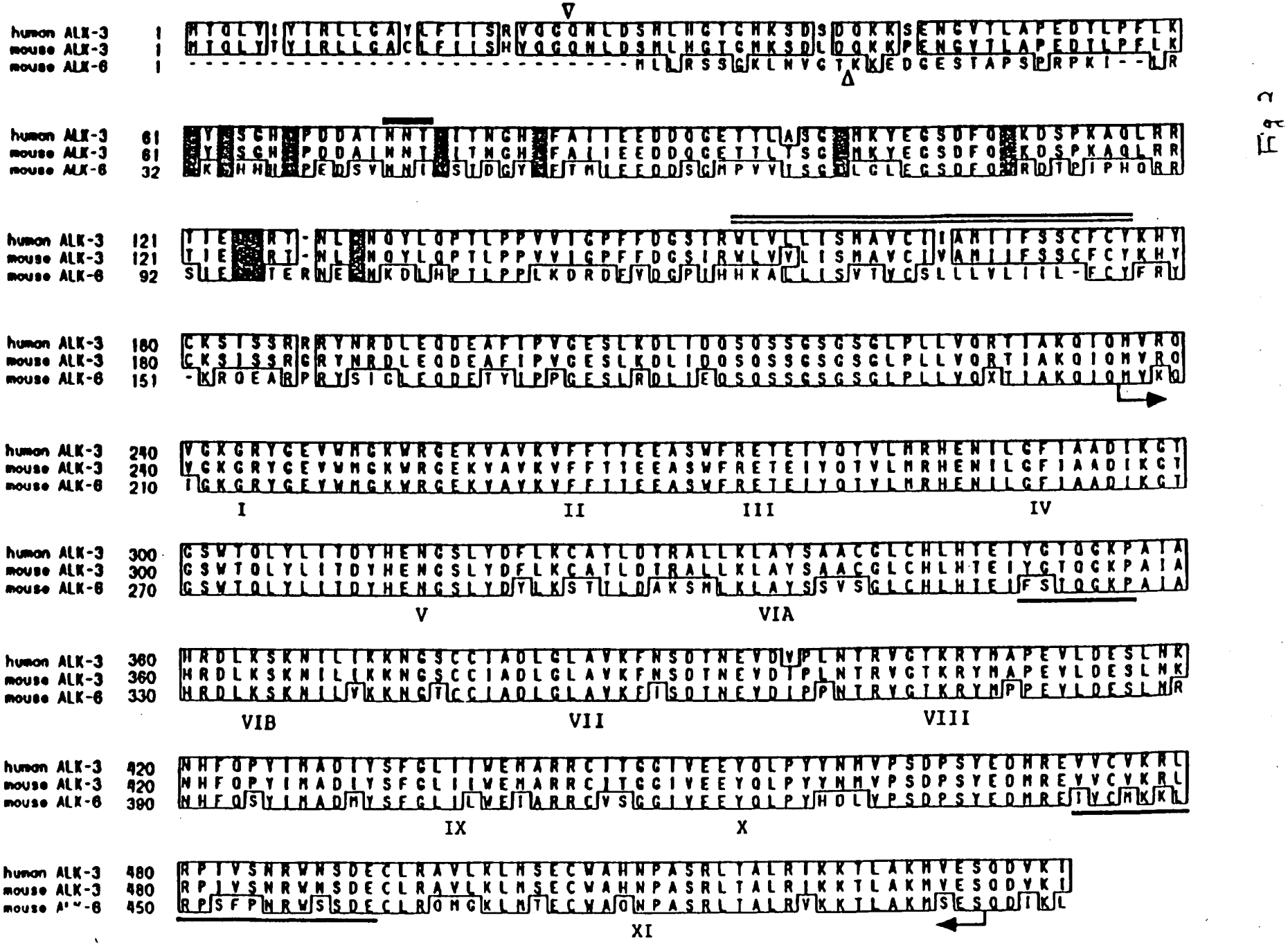


Fig. 1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mouse ALK-3 1
mouse ALK-6 1
human ALK-3 61
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mouse ALK-6 32
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mouse ALK-6 92
human ALK-3 180
mouse ALK-3 180
mouse ALK-6 151
human ALK-3 240
mouse ALK-3 240
mouse ALK-6 210
I
II
III
IV
human ALK-3 300
mouse ALK-3 300
mouse ALK-6 270
V
VIA
human ALK-3 360
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mouse ALK-6 330
VIB
VII
VIII
human ALK-3 420
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IX
X
human ALK-3 480
mouse ALK-3 480
mouse ALK-6 450
XI

Fig 2

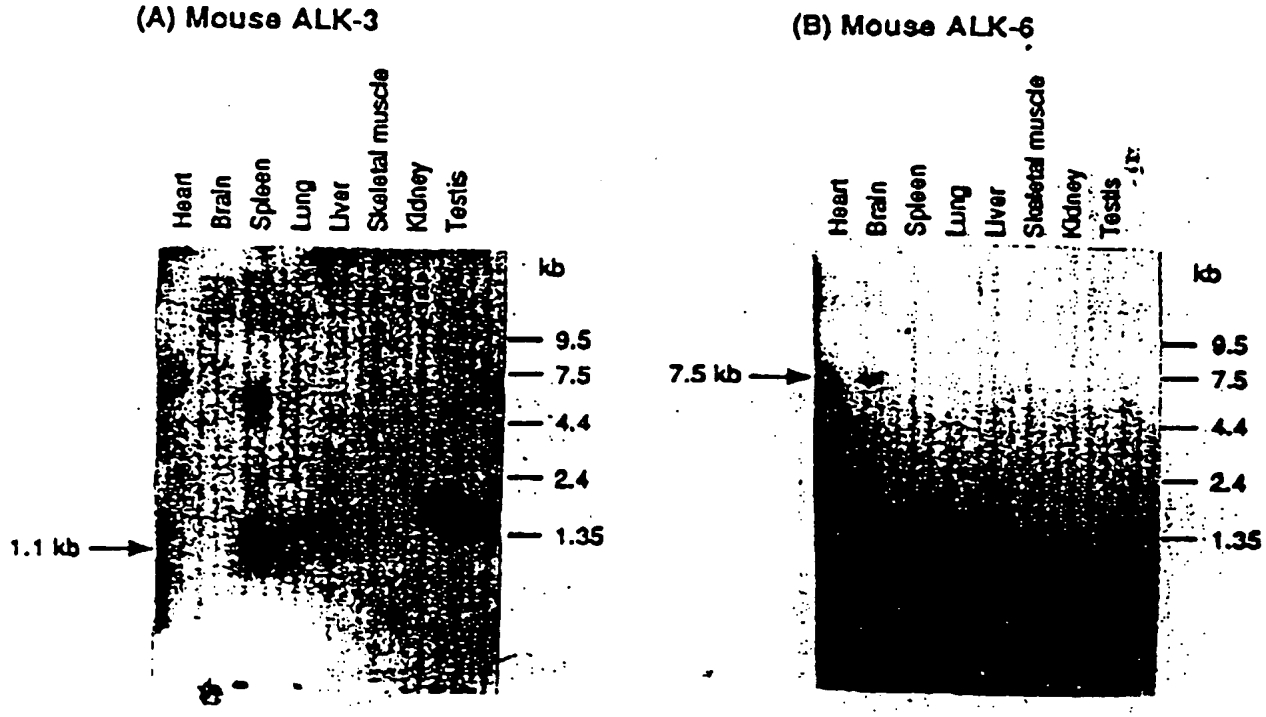
(A) Mouse ALK-3
Heart
Brain
Spleen
Lung
Liver
Skeletal muscle
Kidney
Testis
kb
9.5
7.5
4.4
2.4
1.35
1.1 kb
(B) Mouse ALK-6
Heart
Brain
Spleen
Lung
Liver
Skeletal muscle
Kidney
Testis
kb
9.5
7.5
4.4
2.4
1.35
7.5 kb

Fig. 3

ALK-4 sequence


```
ATGGCGGAGTCGGCCGGAGCCTCCTCCTTCTTCCCCCTTGTTGTCCTCCTGCTCGCCGGCAGCGGCGGGT 70
   A  E  S  A  G  A  S  S  F  F  P  L  V  V  L  L  L  A  G  S  G  G

GGGCCCCGGGGGGTCCAGGCTCTGCTGTGTGCGTGCACCAGCTGCCTCCAGGCCAACTACACGTGTGA 140
S  G  P  R  G  V  Q  A  L  L  C  A  C  T  S  C  L  Q  A  N  Y  T  C  E

GACAGATGGGGCCTGCATGGTTTCCATTTTCAATCTGGATGGGATGGAGCACCATGTGCGCACCTGCATC 210
  T  D  G  A  C  M  V  S  I  F  N  L  D  G  M  E  H  H  V  R  T  C  I

CCCAAAGTGGAGCTGGTCCCTGCCGGGAAGCCCTTCTACTGCCTGAGCTCGGAGGACCTGCGCAACACCC 280
 P  K  V  E  L  V  P  A  G  K  P  F  Y  C  L  S  S  E  D  L  R  N  T

ACTGCTGCTACACTGACTACTGCAACAGGATCGACTTGAGGGTGCCCAGTGGTCACCTCAAGGAGCCTGA 350
H  C  C  Y  T  D  Y  C  N  R  I  D  L  R  V  P  S  G  H  L  K  E  P  E

GCACCCGTCCATGTGGGGCCCGGTGGAGCTGGTAGGCATCATCGCCGGCCCGGTGTTCCTCCTGTTCCTC 420
  M  P  S  M  W  G  P  V  E  L  V  G  I  I  A  G  P  V  F  L  L  F  L

ATCATCATCATTGTTTTCCTTGTCATTAACTATCATCAGCGTGTCTATCACAACCGCCAGAGACTGGACA 490
 I  I  I  I  V  F  L  V  I  N  Y  H  Q  R  V  Y  H  N  R  Q  R  L  D

TGGAAGATCCCTCATGTGAGATGTGTCTCTCCAAAGACAAGACGCTCCAGGATCTTGTCTACGATCTCTC 560
M  E  D  P  S  C  E  M  C  L  S  K  D  K  T  L  Q  D  L  V  Y  D  L  S

CACCTCAGGGTCTGGCTCAGGGTTACCCCTCTTTGTCCAGCGCACAGTGGCCCGAACCATCGTTTTACAA 630
  T  S  G  S  G  S  G  L  P  L  F  V  Q  R  T  V  A  R  T  I  V  L  Q

GAGATTATTGGCAAGGGTCGGTTTGGGGAAGTATGGCGGGGCCGCTGGAGGGGTGGTGATGTGGCTGTGA 700
 E  I  I  G  K  G  R  F  G  E  V  W  R  G  R  W  R  G  G  D  V  A  V

AAATATTCTCTTCTCGTGAAGAACGGTCTTGGTTCAGGGAAGCAGAGATATACCAGACGGTCATGCTGCG 770
K  I  F  S  S  R  E  E  R  S  W  F  R  E  A  E  I  Y  Q  T  V  M  L  R

CCATGAAAACATCCTTGGATTTATTGCTGCTGACAATAAAGATAATGGCACCTGGACACAGCTGTGGCTT 840
  H  E  N  I  L  G  F  I  A  A  D  N  K  D  N  G  T  W  T  Q  L  W  L

GTTTCTGACTATCATGAGCACGGGTCCCTGTTTGATTATCTGAACCGGTACACAGTGACAATTGAGGGGA 910
 V  S  D  Y  H  E  H  G  S  L  F  D  Y  L  N  R  Y  T  V  T  I  E  G

TGATTAAGCTGGCCTTGTCTGCTGCTAGTGGGCTGGCACACCTGCACATGGAGATCGTGGGCACCCAAGG 980
M  I  K  L  A  L  S  A  A  S  G  L  A  H  L  H  M  E  I  V  G  T  Q  G

GAAGCCTGGAATTGCTCATCGAGACTTAAAGTCAAAGAACATTCTGGTGAAGAAAAATGGCATGTGTGCC 1050
  K  P  G  I  A  H  R  D  L  K  S  K  N  I  L  V  K  K  N  G  M  C  A

ATAGCAGACCTGGGCCTGGCTGTCCGTCATGATGCAGTCACTGACACCATTGACATTGCCCCGAATCAGA 1120
 I  A  D  L  G  L  A  V  R  H  D  A  V  T  D  T  I  D  I  A  P  N  Q

GGGTGGGGACCAAACGATACATGGCCCCTGAAGTACTTGATGAAACCATTAATATGAAACACTTTGACTC 1190
R  V  G  T  K  R  Y  M  A  P  E  V  L  D  E  T  I  N  M  K  H  F  D  S

CTTTAAATGTGCTGATATTTATGCCCTCGGGCTTGTATATTGGGAGATTGCTCGAAGATGCAATTCTGGA 1260
  F  K  C  A  D  I  Y  A  L  G  L  V  Y  W  E  I  A  R  R  C  N  S  G

GGAGTCCATGAAGAATATCAGCTGCCATATTACGACTTAGTGCCCTCTGACCCTTCCATTGAGGAAATGC 1330
 G  V  H  E  E  Y  Q  L  P  Y  Y  D  L  V  P  S  D  P  S  I  E  E  M

GAAAGGTTGTATGTGATCAGAAGCTGCGTCCCAACATCCCCAACTGGTGGCAGAGTTATGAGGCACTGCG 1400
R  K  V  V  C  D  Q  K  L  R  P  N  I  P  N  W  W  Q  S  Y  E  A  L  R

CGTGATGGGCAAGATGATGCGAGAGTGTTGGTATGCCAACGGCGCAGCCCGCCTGACGGCCCTGCGCATC 1470
  V  M  G  K  M  M  R  E  C  W  Y  A  N  G  A  A  R  L  T  A  L  R  I
```

CCCTCTCCCAGCTCAGCGTGCAGGAAGACGTGAAGATCTAACTGCTCCCTCTCTCCACACGGA 1540
. . T L S Q L S V Q E D V K I .

GCTCCTGGCAGCGAGAACTACGCACAGCTGCCGCGTTGAGCGTACGATGGAGGCCTACCTCTCGTTTCTG 1610

CCCAGCCCTCTGTGGCCAGGAGCCCTGGCCCGCAAGAGGGACAGAGCCCGGGAGAGACTCGCTCACTCCC 1680

ATGTTGGGTTTGAGACAGACACCTTTTCTATTTACCTCCTAATGGCATGGAGACTCTGAGAGCGAATTGT 1750

GTGGAGAACTCAGTGCCACACCTCGAACTGGTTGTAGTGGGAAGTCCCGCGAAACCCGGTGCATCTGGCA 1820

CGTGGCCAGGAGCCATGACAGGGGCGCTTGGGAGGGGCCGGAGGAACCGAGGTGTTGCCAGTGCTAAGCT 1890

GCCCTGAGGGTTTCCTTCGGGGACCAGCCCACAGCACACCAAGGTGGCCCGGAAGAACCAGAAGTGCAGC 1960

CCCTCTCACAGGCAGCTCTGAGCCGCGCTTTCCCCTCCTCCCTGGGATGGACGCTGCCGGGAGACTGCCA 2030

GTGGAGACGGAATCTGCCGCTTTGTCTGTCCAGCCGTGTGTGCATGTGCCGAGGTGCGTCCCCCGTTGTG 2100

CCTGGTTCGTGCCATGCCCTTACACGTGCGTGTGAGTGTGTGTGTGTCTCTGTAGGTGCGCACTTACCTG 2170

CTTGAGCTTTCTGTGCATGTGCAGGTCGGGGGTGTGGTCGTCATGCTGTCCGTGCTTGCTGGTGCCTCTT 2240

TTCAGTAGTGAGCAGCATCTAGTTTCCCTGGTGCCCTTCCCTGGAGGTCTCTCCCTCCCCCAGAGCCCCT 2310

CATGCCACAGTGGTACTCTGTGT 2333